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Cytogenomic Analysis of Four Philippine Medicinal Plants Using Fluorescence *In Situ* Hybridization and Flow Cytometry

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Chromosome numbers, karyotype, and genome size provide fundamental information for genome sequencing and breeding studies. Here, we analyzed the karyotype and genome size of four Philippine medicinal plants – namely *Carica papaya* (papaya), *Citrus microcarpa* (calamansi), *Cymbopogon citratus* (tanglad), and *Moringa oleifera* (malunggay). We performed a triple-color fluorescence *in situ* hybridization to detect the chromosomal distribution of the 5S and 35S rDNA and the *Arabidopsis*-type telomeric repeat. *Citrus microcarpa* (2*n* = 18, 2C = 0.95 pg) had one pair of 5S and 35S rDNA and a hemizygous 35S rDNA in the centromeric region of Chromosome 3. *Carica papaya* (2*n* = 18, 1.08 pg) had five pairs of 5S rDNA and one pair of 35S rDNA. *Cymbopogon citratus* (2*n* = 60, 3.79 pg) had four pairs of 5S rDNA and one pair of 35S rDNA. *Moringa oleifera* (2*n* = 28, 1.36 pg) had two pairs of 5S rDNA and five pairs of 35S rDNA. The telomeric repeat was detected in the chromosome termini of all chromosomes. Here, we present basic cytogenomics information on these four species, which can be useful for genomics, breeding, and evolutionary research. This approach can be replicated to gather cytogenomic information on endemic Philippine plants for genomics and conservation.

Keywords: cytogenomics, fluorescence *in situ* hybridization, genome size, karyotype, repeat distribution

INTRODUCTION

The Philippine archipelago is home to ~ 5% of the world's flora or more than 10,000 plant species, of which 45–60% are endemic (Malayang 2021), making the Philippines one of the mega biodiverse countries in the world (de Leon *et al*. 2019). Many of these species are endemic and economically important, but unfortunately, many are also endangered due to excessive poaching and deforestation (Coritico and Amoroso 2020; de Leon *et al*. 2019). Plant systematics and conservation efforts have been intensified to provide a comprehensive reference of Philippine plant species and attempt to reverse the effects of deforestation (Coritico and Amoroso 2020; Paclibar and Tadiosa 2020).

Genomics research can facilitate the sustainable use of Philippine plant species and improve agricultural production. For example, promoting genomics research in the Philippines has reaped benefits in the coconut

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industry (Lantican *et al*. 2019). With the high endemicity of Philippine plant species, it is of great national interest to study these species for conservation and sustainable consumption for food, medicine, and other industries. To complement genomics research, cytogenomic information such as chromosome number, genome size, and karyotype are essential to facilitate genome assemblies and understand species relationships.

One of the basic techniques used in modern cytogenomics is fluorescence *in situ* hybridization (FISH). FISH is a powerful technique to determine the chromosomal location of DNA probes. Oligonucleotide probes from repetitive elements have been widely used to identify homologous chromosomes and build karyotypes and phylogenetic relationships of closely related species to study genome organization and ploidy in plants (Bishop 2010; Jiang *et al*. 2017; Pellerin *et al*. 2019; Ta *et al*. 2021; Waminal *et al*. 2017, 2018). FISH using the highly conserved repeat sequences of 5S rDNA, 35S rDNA, and the *Arabidopsis*-type telomere (TTTAGGG) allows higher cytogenetic resolution than conventional staining of chromosomes. This enables FISH to better elucidate homologous chromosomes, repeat distribution, and phylogenetic relationships (Jiang *et al*. 2017; Waminal *et al*. 2018). In addition, FISH using pre-labeled oligonucleotide probes (PLOPs) has now been used for newly identified repeats of several species to clearly discriminate sub-genomes (Campomayor *et al*. 2021) or individual chromosomes (Ta *et al*. 2021; Waminal *et al*. 2017).

Along with genome size data, chromosome information can facilitate evolutionary studies (Doležel *et al*. 2007). These data are also useful in genomics and breeding studies (Doležel *et al*. 2007). Here we provide a model system for generating cytogenomic data of Philippine plants by analyzing the chromosome number, karyotype, and genome size of four Philippine medicinal plants that have ethnopharmacological properties and are widely common in the Philippines – namely *Carica papaya* (papaya), *Citrus microcarpa* (calamansi), *Cymbopogon citratus* (tanglad), and *Moringa oleifera* (malunggay). These economically important plants are cultivated nationwide with increased production annually (Magdalita and San Pascual 2023). Thus, a cytogenomic study of these plants will be an initial approach for genomics research and plant improvement.

MATERIALS AND METHODS

Plant Materials

Individual plants of *C. papaya*, *C. macrocarpa*, *C. citratus*, and *M. oleifera* were grown in a greenhouse. Prior to chromosome spread preparation, healthy young roots from potted plants were harvested in the morning (09:00–10:00 AM), pretreated with 0.6–0.8 MPa nitrous oxide (N_2O) to arrest metaphase cells, and then soaked in ice-cold water overnight to enhance chromosome spreading. The following day, roots were fixed in 1:3 (v/v) aceto-ethanol or Carnoy's solution for 4 h and stored in 70% ethanol at 4ºC until further use (Peniton *et al*. 2022).

Chromosome Spread Preparation

Metaphase chromosome spreads were prepared following our previous report (Campomayor *et al*. 2021). Briefly, the root caps and the non-actively dividing region of the roots were removed under a dissecting microscope, and only the whitish tips, which contain the actively dividing meristematic cells, were enzymatically digested for 90 min at 37 ℃ with 1%: 2% (w/v) ratio of pectolyase and cellulase. Then the enzymes were removed, and the protoplasts were suspended in a pre-chilled Carnoy's solution for 5 min, centrifuged, and quickly resuspended in the 9:1 (v/v) aceto-ethanol solution before being mounted onto pre-warmed slides in a humid chamber, which facilitate chromosome spreading. Finally, the slides were air-dried, fixed in 2% formaldehyde for 5 min, and dehydrated in a series of increasing ethanol concentrations (70, 90, and 100%) for 3 min each.

Fluorescence *In Situ* Hybridization (FISH)

The PLOP probes for the 5S rDNA, 35S rDNA, and the *Arabidopsis*-type telomeric repeat were designed by Waminal *et al.* (2018). The 5′ ends of each PLOP were modified with FAM (5S rDNA), Cy3 (35S rDNA), and Texas Red (*Arabidopsis*-type telomere) purchased from Bioneer Corporation (South Korea; https://www.bioneer.co.kr/).

The FISH procedure was carried out based on previous publications (Pellerin *et al*. 2019; Waminal *et al*. 2018). Briefly, a 40-µl FISH hybridization master mix containing 50% formamide, 10% dextran sulfate, 2x SSC, and 50 ng uL^{-1} of each probe was pipetted onto fixed slides with chromosome spreads. After being denatured at 80 ℃ for 5 min, the probes were allowed to hybridize overnight at 37 ℃ in a humid chamber. The next day, the slides were sequentially washed in 2× SSC at RT for 10 min, 0.1× SSC at 42 ℃ for 25 min, and 2× SSC at RT for 5 min and then dehydrated in a series of ethanol concentrations. Slides were air-dried and counterstained with 1:100 4',6-diamidino-2-phenylindole (DAPI) (100 µg mL^{-1} stock) to Vectashield (Vector H1000, Vector Labs, USA).

Karyotyping

The chromosome counts from each plant species were confirmed from at least 30 metaphase spreads obtained

from several prepared slides. Five well-condensed metaphase chromosomes were FISH analyzed. Images of chromosomes and FISH signals were obtained under an Olympus BX53 fluorescence microscope (Olympus Co., Tokyo, Japan) equipped with a DFC365 FS CCD camera (Leica Microsystems, Wetzlar, Germany) using an oil immersion objective (×100 magnification). Raw images were analyzed and enhanced in Adobe Photoshop CS6 (Adobe System Inc., California, USA). The chromosome lengths from five metaphase chromosomes were measured using ImageJ v1.51k and were classified according to the existing criteria (Levan *et al*. 1964). Homologous chromosomes were paired based on FISH signal distribution and centromeric position. The chromosomes were arranged by decreasing order of chromosome length (Pellerin *et al*. 2019), and then the ideograms were generated using Adobe Illustrator CS6 (Adobe System Inc., California, USA).

Estimation of DNA Content

The genomic sizes were measured from the isolated young leaf nuclei of each plant (Doležel *et al*. 2007). *Glycine max* (2C = 2.50 pg) was used as the internal reference standard (Doležel *et al*. 2007). Briefly, the young leaves from the plant sample and the internal reference were chopped simultaneously at ~ 5 chops per second for 30 s using a sharp razor blade in a glass petri dish containing 500-µL LB01 buffer. To separate the nuclei and the cellular debris, the lysate was filtered sequentially through a 50 µm x 30 µm nylon mesh. Then, the nuclei were stained with 50 µg mL^{-1} propidium iodide, and the RNA contaminants were degraded in 10 µg mL^{-1} RNAse. The suspension was set on ice for ~ 2 min before flow cytometric analysis.

DNA content was analyzed using a CytoFLEX flow cytometer equipped with a 50-mW 488-nm solid-state diode laser and CytExpert v2.3 software (Beckman Coulter Inc., USA). About 5,000–10,000 events (nuclei counts) were recorded to build the histograms. To estimate the 2C DNA content, the mean 2C peak of the experimental sample was divided by the mean 2C peak of the internal reference and then multiplied by the 2C value of the reference. To calculate the genome size in bp units, the 1C DNA content value of the samples was multiplied by 0.978 x 10^9 bp (Doležel *et al*. 2007; Doležel and Bartoš 2005).

RESULTS

Cytogenetic Features

The karyological features of the four medicinal plants are shown in Table 1. *C. papaya* had a chromosome complement of 2*n* = 18, with chromosome length ranging from 2.55 ± 0.08 to 3.31 ± 0.04 µm and a total length of 52.01 ± 2.38 µm. *C. microcarpa* had a chromosome complement of 2*n* = 18 with chromosome length ranging from 2.39 ± 0.13 to 3.49 ± 0.29 µm and a total length of 51.48 ± 2.15 µm. *C. citratus* had a chromosome complement of 2*n* = 60 with chromosome length ranging from 2.04 ± 0.07 to 4.24 ± 0.10 µm and a total length of 182.14 ± 8.85 µm. Lastly, *M. oleifera* had a chromosome complement of 2*n* = 28 with chromosome length ranging from 2.60 ± 0.31 µm to 4.58 ± 0.27 µm and a total length of 99.33 ± 7.59 µm. The chromosome complement of all sampled species was composed of metacentric homologous chromosomes except on Chromosomes 1 and 14 of *M. oleifera*, which exhibited a subtelomeric chromosome type.

The 5S rDNA, 35S rDNA, and the *Arabidopsis*-type telomeric repeats had different loci counts among the four species analyzed (Figure 1). In *C. papaya*, two pairs of intense and three pairs of weak 5S rDNA signals were observed in the short arms of Chromosomes 2 and 7, and Chromosomes 3, 6, and 9, respectively. The 35S rDNA signal was located on the nucleolar organizing region (NOR) of the short arm of Chromosome 1 (Figures 1A, 2, and 3). In *C. macrocarpa*, one hemizygous 35S rDNA locus was observed in the paracentromeric region of the short arm of Chromosome 3, whereas proximal and distal

Table 1. Chromosome constitution, FISH karyotype, and genome size analysis of the four medicinal plants.

Species name	Local name	Chr. no. (2*n*)	Chromosome length (µm)			Chr. type	FISH signals (2*n*)			DNA content	
			Shortest	Longest	Total		5S rDNA	45S rDNA	Telomeric repeat	2C, (pg)	1C, (pg)
C. microcarpa	*Calamansi*	18	2.39 ± 0.13	3.49 ± 0.29	51.48 ± 2.15	m	2	3	t	0.95 ± 0.01	0.48
C. papaya	Papaya	18	2.55 ± 0.08	3.31 ± 0.04	52.01 ± 2.38	m	10	2	t	1.08 ± 0.04	0.53
C. citratus	Lemongrass	60	2.04 ± 0.07	4.24 ± 0.10	182.14 ± 8.85	m	8	2	t	3.79 ± 0.01	1.90
M. oleifera	*Malunggay*	28	2.60 ± 0.31	4.58 ± 0.27	99.33 ± 7.59	m st (chr. 1,14)	4	10	t	1.36 ± 0.03	0.68

[m] metacentric; [st] subtelomeric; [t] terminal

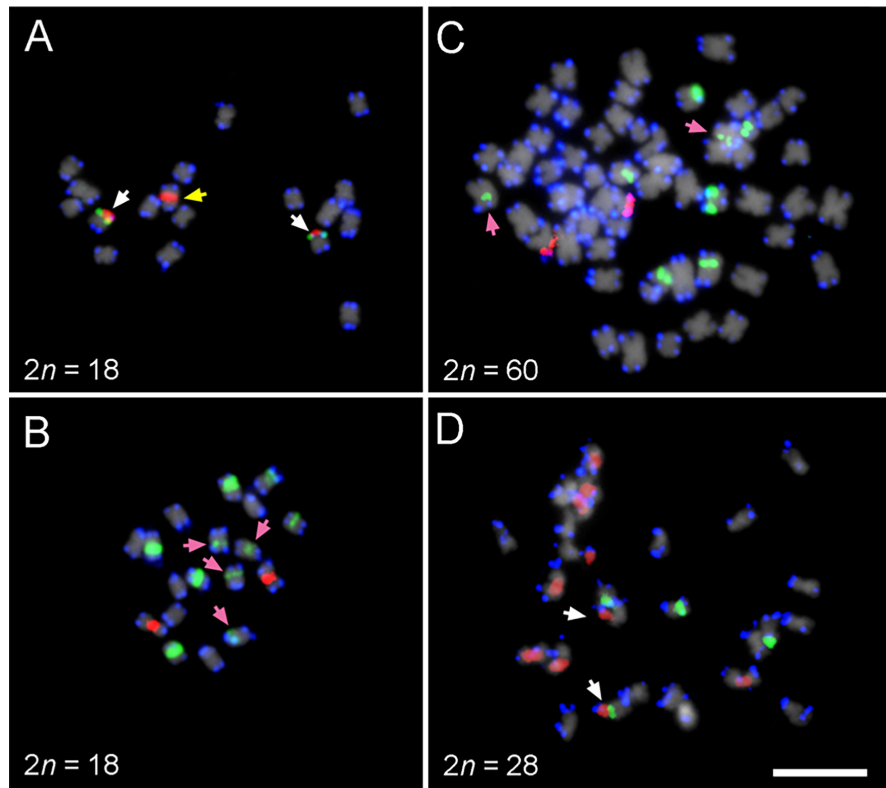

Figure 1. Triple-color FISH karyotype on four medicinal plant species in the Philippines: [A–D] FISH metaphase karyotype obtained from [A] *C. microcarpa* (2*n* = 18), [B] *C. papaya* (2*n* = 18), [C] *C. citratus* (2*n* = 60), and [D] *M. oleifera* (2*n* = 28), respectively. The PLOP-FISH used were the 5S rDNA (green), 45S rDNA (red), and the *Arabidopsis*-type telomere (blue). The chromosome counts have been confirmed from at least 10 metaphase spreads from each species. The yellow, pink, and white arrows point to the chromosome having a hemizygous signal of 45S rDNA, weak signals of 5S rDNA, and the detection of both 5S and 45S rDNA signals, respectively. Bar = 10 µm.

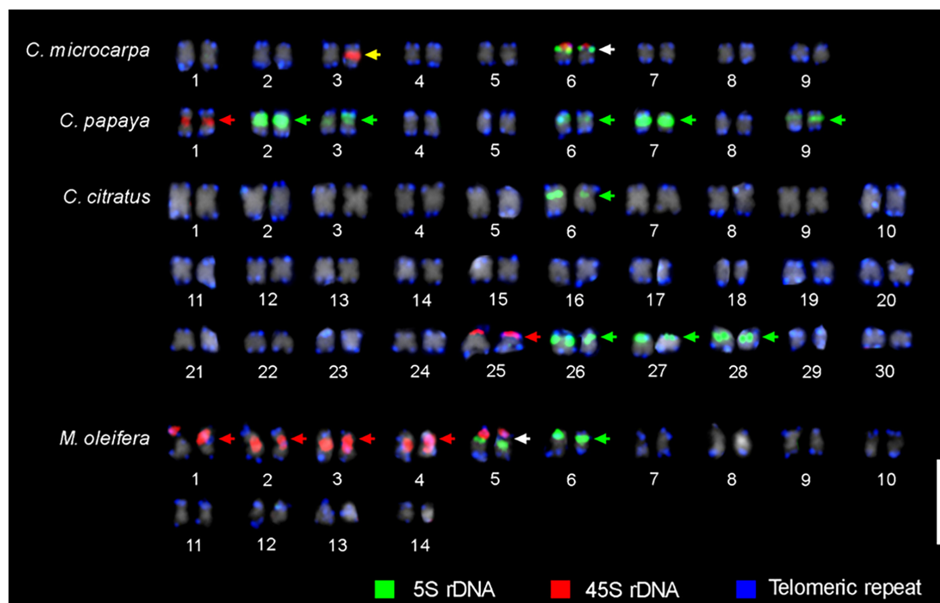

Figure 2. FISH karyogram of the four Philippine medicinal plants. The chromosomes of each species were arranged in decreasing order and paired based on the centromeric position and FISH probe distribution. The green, red, yellow, and white arrows point to the chromosome having the 5S rDNA, 45S rDNA, hemizygous signal of 45S rDNA, and the detection of both 5S and 45S rDNA signals, respectively. Bar = 10 µm.

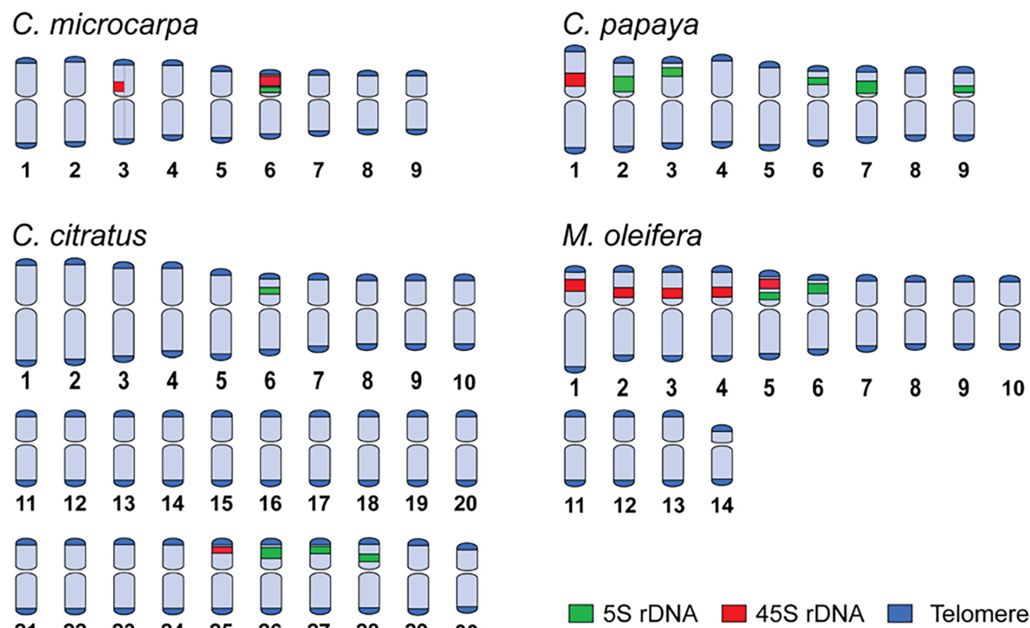

Figure 3. FISH ideogram showing the FISH probe distribution. The chromosomes of each species were arranged in decreasing order. The green, red, and blue colors indicate the position of the 5S rDNA, 45S rDNA, and telomeric repeats, respectively.

signals of 5S and 35S rDNA were detected in the short arm of chromosome 6 (Figures 1B, 2, and 3), respectively. In *C. citratus*, four pairs of 5S rDNA signals were detected in the short arm of Chromosomes 6, 26, 27, and 28. The 35S rDNA was located in the subtelomeric region of the short arm of chromosome 25 (Figures 1C, 2, and 3). In *M. oleifera,* the two pairs of 5S rDNA signals were detected in the short arm of Chromosomes 5 and 6. There were five pairs of 35S rDNA signals spotted in the NOR region of Chromosomes 1, 2, 3, 4, and 5. In Chromosome 5, the 35S rDNA is distal to the 5S rDNA locus in the short arm (Figures 1D, 2, and 3). The telomeric repeats were detected in the canonical termini chromosomes of the four species. FISH karyograms showing paired homologous chromosomes in decreasing order (Figure 2) with their corresponding ideograms (Figure 3) summarize the distribution of the cytogenetic markers in the root metaphase chromosomes of the four species.

Estimated Genome Size

The G0/G1 peak of *G. max* internal reference was positioned at Channel 120, and the DNA content of the four species was calculated relative to the reference peak (Figure 4). The estimated 2C genome sizes of *C. papaya*, *C. microcarpa*, *C. citratus*, and *M. oleifera* were 3.79, 1.08, 0.95, and 1.36 pg. This is equivalent to the 1C value of 1.90, 0.53, 0.48, and 0.68 pg, respectively (Table 1).

DISCUSSION

Chromosome numbers, karyotype, and genome size are invaluable information for evolution, genomics, and breeding research. Here, we provided the chromosome number, karyotype, and genome size information for four Philippine medicinal plants.

Our chromosome counts corresponded with previous reports: 2n = 18 for *C. papaya* (Fabiane *et al*. 2008) and *C. microcarpa* (He *et al*. 2020; Pedrosa *et al*. 2000), 2n = 60 for *C. citratus* (Vyshali *et al*. 2017), and 2n = 28 for *M. oleifera* (Zhang *et al*. 2020). Except for *C. citratus*, which is a hexaploid (Vyshali *et al*. 2017), the other three species are diploids (Fabiane *et al*. 2008; He *et al*. 2020; Pedrosa *et al*. 2000; Zhang *et al*. 2020). While chromosome counts have been reported for *C. papaya*, *C. microcarpa*, *C. citratus*, and *M. oleifera* (Fabiane *et al*. 2008; He *et al*. 2020; Pedrosa *et al*. 2000; Vyshali *et al*. 2017; Zhang *et al*. 2020), here we report their karyotype based on triple-color PLOP-FISH.

We could observe a hemizygous 35S rDNA signal in *C. macrocarpa* Chromosome 3. This hemizygous locus is not unique to *C. microcarpa*, as this pattern has been observed also in its related species, *C. reticulata* (He *et al*. 2020). This pattern reflects the hybrid nature of *C. reticulata*, as revealed by the genome sequence data, where there is an introgression of *C. maxima* into *C. reticulata* (Wu *et al*. 2018). Possibly, *C. microcarpa* is also a product of homoploid hybridization. Further cytogenetic and phylogenomic studies are needed to understand and trace

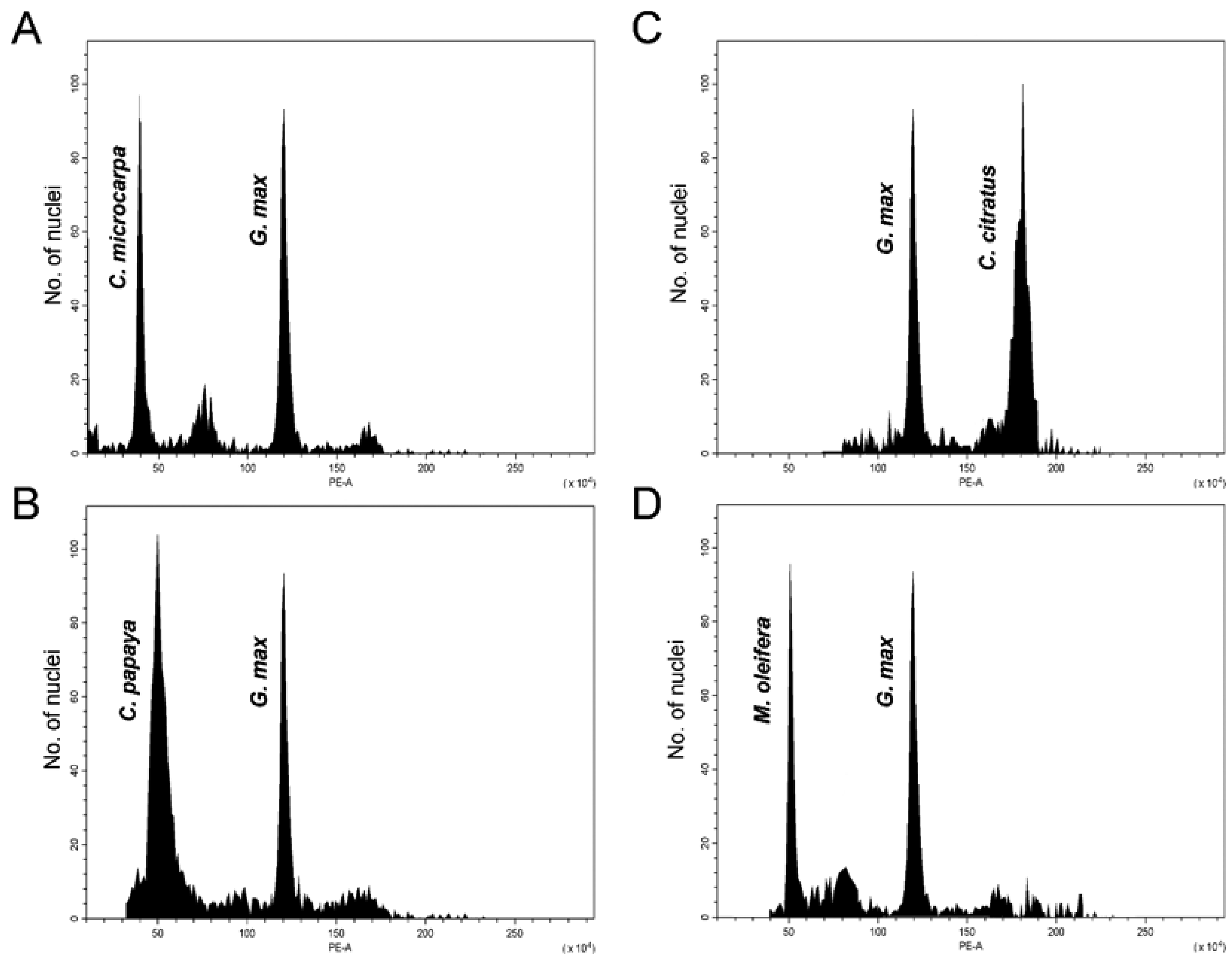

Figure 4. Flow histogram constructed from the four sampled plants and the *G. max* as the internal reference standard. The estimation of relative 2C DNA content from the four sampled plants using *G. max* as the internal reference standard. Each peak was positioned on Channel 40 for *C. microcarpa* (A), Channel 50 for *C. papaya* (B), Channel 180 for *C. citratus* (C), and Channel 50 for *M. oleifera* (D), whereas the internal reference standard was positioned in Channel 120.

its origin, as the taxonomic placement of many species or accessions within the genus *Citrus* is often controversial (Pedrosa *et al.* 2000; Song *et al.* 2023; Wu *et al.* 2018).

Although *C. citratus* ($2n = 60$) is regarded as a hexaploid based on chromosome count with a base chromosome number of $x = 10$ (Thakur *et al*. 2021), the 35S and 5S rDNA FISH signals did not correspond to the putative ploidy. We observed only one 35S rDNA locus, contrary to an expected three when loci count is additive after polyploidization. Moreover, we also detected only four 5S rDNA loci, which may indicate a loss of two loci if each donor genome has two. Conversely, it may have gained a 5S rDNA locus after polyploidization. Nevertheless, a non-additive count of rDNA loci is often observed in polyploids as these loci are very rapidly evolving, with some loci even becoming eliminated or gained in a single generation (Heslop-Harrison and Schwarzacher 2011). At least one locus is enough for each 35S and 5S rDNA to compensate for proper ribosomal function (Alvarez and Wendel 2003). Thus, polyploids are less stringent in losing redundant rDNA loci (Garcia *et al*. 2017).

Our investigation into chromosome counts in *C. papaya* agreed with the findings of Fabiane *et al*. (2008). However, our FISH analysis on 5S rDNA differs from their observation. In our study, we detected the 5S rDNA loci across five homologous pairs, distinguishing two pairs with intense signals and three pairs with weaker signals. Whereas in their report, they only observed signals in two pairs of chromosomes. This discrepancy could stem from our utilization of the pre-labeled oligoprobes, a method noted for its enhanced efficiency in detecting rDNA signals compared to conventional FISH using nucleotide analog conjugated with either hapten or fluorochrome (Waminal *et al*. 2018). Alternatively, different accessions of the same species could also have different numbers of rDNA loci (He *et al*. 2022).

FISH report on the number of rDNA loci in *C. macrocarpa* is similar to the previous study (Song *et al*. 2023). Its hemizygous 35S rDNA signal observed in Chromosome 3 may be due to unequal crossing over (Pellerin *et al*. 2019), chromosomal rearrangement (Orooji *et al*. 2022), reduction of repeat size (Rocha *et al*. 2015) and epigenetic silencing (Rossello *et al*. 2022) that eventually lead to unbalanced copy numbers of the whole tandem repeat unit between homologous chromosomes. Our study also presents the first FISH data of *M. oleifera*, revealing a notable abundance of 35S rDNA loci. This abundance could indicate heightened protein synthesis activity (Rocha *et al*. 2015; Rossello *et al*. 2022), genomic variability, and detection of both functional and non-functional copies of the gene (Orooji *et al*. 2022).

Three of the four species investigated in this study have small 1C genome sizes based on the definition by Schubert and Vu (2016), whereas *C. citratus* has a medium-sized genome. Conversely, when comparing the monoploid genome sizes, considering that *C. citratus* is a hexaploid, all four species have small monoploid genome sizes.

Our genome size data in *M. oleifera* and *C. citratus* are closely similar to the previous reports of Leone *et al*. (2015) with 1.2 pg and Jagadeeshan *et al*. (2019) with 3.51 pg. However, our genome size for *C. papaya* with 1.08 pg is far different from the 0.65 pg report of Araújo *et al*. (2010). This variance can be attributed to our utilization of *G. max* as the internal reference standard recommended by Doležel and Bartoš (2005). Unlike pseudo-internal standardization, our approach involved staining and analyzing both the reference standard (*G. max*) and the unknown sample (*C. papaya*) together. The selection of a genetically stable internal reference, calibrated in absolute units, plays a pivotal role in accurately estimating nuclear DNA content (Dolezel *et al*. 2007). Additionally, the observed increase in genome size in our study may account for the four additional 35S rDNA loci as weak signals in our FISH data. Alternatively, this difference in genome size and the FISH pattern in *C. papaya* likely reflects a difference in genotype.

Chromosome number and genome size data are important in comparative studies within a group of taxa (Bishop 2010; Jiang *et al*. 2017). This information allows a deeper understanding of the chromosomal basis of species evolution within a group, which cannot be achieved by either chromosome number or genome size data alone. This is because chromosome number and genome size often do not correlate positively (Raca *et al*. 2023). Genome size increases while chromosome number is reduced in a phylogenetically anchored clade may indicate descending dysploidy with an associated burst of repetitive elements (Raca *et al*. 2023). In addition, genome size and chromosome number information are important in genome sequencing and assembly. This information enables one to select the best sequencing strategy and to interpret genome assembly output.

CONCLUSION

We have presented a triple-color PLOP-FISH karyotype and genome size estimation of four Philippine medicinal plants. Considering the high rate of endemism in the Philippines (Coritico and Amoroso 2020), cytogenomic data can help evolutionary biology researchers understand insular species diversification. Moreover, genomics research can also benefit from a cytogenomic database of Philippine plants.

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